



Published in final edited form as:

J Immunol. 2010 March 15; 184(6): 3087–3097. doi:10.4049/jimmunol.0901167.

CC Chemokine Ligand 3 Overcomes the Bacteriocidal and Phagocytic Defect of Macrophages and Hastens Recovery from Experimental Otitis Media in TNF^{-/-} Mice

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Abstract

Innate immune mechanisms are crucial in defense against bacterial illnesses in humans, as evidenced by abnormal antibacterial responses due to defects in TLR signaling, seen in children with MyD88 or IL-1R-associated kinase 4 deficiency. Otitis media (OM) is the most common disease of childhood, and the role of innate immune molecules in this disorder remains unclear. In a murine model of OM, we show that, in the absence of TNF, a key effector of innate immunity, this disease is prolonged after middle ear infection with nontypeable *Haemophilus influenzae* (*NTHi*). In the absence of TNF, mice fail to upregulate both TLRs and downstream genes and proteins, such as CCL3, resulting in defects in both inflammatory cell recruitment and macrophage function. Peritoneal macrophages of mice lacking TNF have a diminished ability to phagocytose and kill *NTHi*, and this defect is partially corrected in vitro by exogenous rTNF. Addition of

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Disclosures

A.F.R. and R.G. are part-time federal government employees at the San Diego Veterans Affairs Medical Center.

The online version of this article contains supplemental material.

Corrections

Leichtle, A., M. Hernandez, J. Ebmeyer, K. Yamasaki, Y. Lai, K. Radek, Y.-H. Choung, S. Euteneuer, K. Pak, R. Gallo, S. I. Wasserman, and A. F. Ryan. 2010. CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF^{-/-} mice. *J. Immunol.* 184: 3087–3097.

The middle initials of the sixth and tenth authors were omitted. The correct names are Katherine A. Radek and Richard L. Gallo. www.jimmunol.org/cgi/doi/10.4049/jimmunol.1090019

rCCL3 alone or in combination with rTNF restores phagocytosis and killing by TNF-deficient macrophages to that of unstimulated wild-type macrophages. In vivo administration of rCCL3 to animals deficient in TNF fully restores the ability to control OM due to *NTHi*, whereas a CCL3-blocking Ab impaired the ability of wild-type mice to recover from OM. Thus, CCL3 is a potent downstream effector of TNF-mediated inflammation in vitro and in vivo. Manipulation of CCL3 and/or TNF may prove to be effective therapeutic approaches in OM or other conditions associated with defective TNF generation.

Otitis media (OM) is the most prevalent disease of childhood and a major public health problem contributing to childhood mortality in less developed countries (1-3). In developed societies, OM accounts for more office visits and drug purchases than any other disease in the first 6 y of life and is the most common reason for outpatient antimicrobial treatment in the United States, with a cost of >\$5 billion per year (4-6). Although acute OM is generally an uncomplicated and self-limited disease, chronic OM is difficult to manage and frequently results in continuous medical treatment and/or multiple surgical procedures with serious sequelae, including recurrent or irreversible pain and long-term hearing loss with consequent delay of language development and academic achievement (7,8).

The precise pathogenesis of OM, considered to be multifactorial, is still poorly understood. However, our group and others have demonstrated that innate immune mechanisms are intimately involved in host defense in murine models of OM. Defense against OM, in a murine model, is defective in the absence of MyD88, TLR2, or TLR4 (9,10). In children, genetic polymorphisms in TLR4 and the TLR coreceptor CD14 are associated with an increased incidence of OM (11-13). Nontypeable *Haemophilus influenzae* (*NTHi*) is a major cause of human disease, including OM (14), and also contributes to exacerbation of chronic obstructive pulmonary disease (15). TLR2 mediates responses upon exposure to bacterial products including peptidoglycan, lipoteichoic acid, lipoarabinomannan, and lipoprotein from Gram-negative bacteria, such as *NTHi* (16-18). Following bacterial ligand binding, TLRs activate various transcription factors, such as NF- κ B (19,20), to mediate expression of genes encoding inflammatory cytokines including *TNF*, *IL-1 β* , *IL6*, and the chemokines *CXCL2* (*MIP-2*) and *CCL3* (*MIP-1 α*) (21,22).

We have reported that the prolonged inflammatory responses of TLR2^{-/-} and TLR4^{-/-} mice during experimental OM are associated with defective induction of TNF (10). TNF is a pleiotropic inflammatory cytokine known to be a major mediator of inflammation (e.g., see Ref. 23), and previous work suggests a pathophysiological role for this mediator in OM. Transtympanic injection of TNF into the middle ears (MEs) of guinea pigs induces inflammation (24), whereas administration of a TNF antagonist, soluble TNF receptor type I, reduces the severity of LPS-, TNF-, and immune-mediated OM in rats (25-28). Polymorphisms in the TNF promoter that alter TNF production have been associated with an increased incidence of OM in children, suggesting that variability in TNF concentrations may alter susceptibility to and/or expression of OM (11,13,29,30).

TNF has been shown to regulate the expression of TLRs and molecules essential for TLR signaling, including NF- κ B, via both positive (21) and negative (31) feedback mechanisms. Thus, TNF may modulate innate immunity and the complex networks of downstream cytokines and chemokines and participate in the orchestration of the inflammatory response by regulating the migration, activation, and biological properties of various effector cells, particularly mononuclear phagocytes, and neutrophilic polymorphonuclear leukocytes (32,33). For example, TNF directly stimulates production of CCL3, a member of the CC chemokine family known to be an important chemotactic and regulatory agent for macrophages (34,35), and TNF also enables neutrophilic migration in response to CCL3

(33). Neutrophil and mononuclear leukocyte migration into the ME cavity is a central feature of the normal response of mice to *NTHi*-mediated OM.

We therefore investigated the role of TNF in a well-established murine model of OM induced by *NTHi* infection of the ME. Our results reveal an important role for TNF in host defense against *NTHi*-induced OM, in part due to defective bacterial phagocytosis and killing by macrophages of TNF^{-/-} mice. In addition, we have uncovered the ability of rCCL3 to overcome defects in phagocytosis and bacterial killing in macrophages obtained from TNF^{-/-} mice in vitro and to restore an appropriate antibacterial response to *NTHi*-induced OM in vivo. These data suggest that migration, phagocytosis, and bacterial killing by macrophages is, in part, dependent upon normal levels of TNF and that CCL3 is an important downstream effector of TNF action in OM.

Materials and Methods

Animals

TNF^{-/-} mice (B6/129S6-Tnf^{tm1Gkl/J}) and age-matched wild-type (WT) controls (B6/129S6 F2 hybrids) were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR2^{-/-} mice, originally generated on a C57BL/6 background by Akira and colleagues (36), were bred in our laboratory. Age-matched C57BL/6 mice were purchased from The Jackson Laboratory. All animals were housed in specific pathogen-free conditions. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center, San Diego, CA.

Bacterial strains and culture conditions

Nontypeable *Haemophilus influenzae* strain 3655 (*NTHi*, biotype II) was used at a concentration of 10⁵ to 10⁶/ml in ~5 µl to induce infection of the ME. The inocula were prepared as previously described (37).

Surgical procedure

Mice were anesthetized with an i.p. injection of 0.1–0.2 ml per 30 g body weight of rodent mixture (13.3 mg/ml ketamine HCl, 1.3 mg/ml xylazine, and 0.25 mg/ml acepromazine). ME bullae were exposed bilaterally by a ventral approach through a midline incision on the neck, and the ME bulla was fenestrated using a 25-gauge needle. A total of 5 × 10²–10³ *NTHi* in ~5 µl was injected into the ME cavity. Excess fluid was absorbed with a sterile cotton swab. The wound was closed, and the skin incision was stapled. The animals received s.c. injections of buprenorphine and lactated Ringers solution postoperatively.

Quantitative real-time PCR

For mucosal gene expression, total RNA from 6–8 *NTHi*-injected MEs from TNF^{-/-}, TLR2^{-/-}, and WT mice per time point, was extracted using the Dynabeads mRNA Direct kit (Invitrogen, Carlsbad, CA). A total of 20 µl mRNA solution was reverse transcribed using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen). Six hours post *NTHi* infection, total RNA from macrophages was extracted using TRIzol, and 1 µg RNA was transcribed with iScript (Bio-Rad, Hercules, CA). Quantitative real-time PCR (qRT-PCR) was performed using 1 µg/µl mRNA and predeveloped Taq-Man assay probes (Applied Biosystems, Foster City, CA) for murine *TNF*, *IL-1β*, *IL6*, *CXCL2*, *CCL3*, *TLR2*, and *TLR4* in the Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. All analyses were performed in triplicate from two to three independent experiments, and the fold induction was calculated using the comparative threshold cycle method as previously described (38). Relative expression levels were normalized to GAPDH and compared with untreated and uninfected mucosa or cells.

Histology

Mice were sacrificed under general anesthesia by intracardiac perfusion with PBS followed by 4% paraformaldehyde (PFA) at 0, 6, and 12 h and 1, 2, 3, 5, 10, 14, and 21 d postinoculation. MEs were dissected, postfixed (4% PFA) overnight, and decalcified (8% EDTA and 4% PFA) for 14 d. ME bullae were embedded in paraffin and sections were cut at 7 μ m. Sections were stained with H&E, digitally recorded, and the grade of OM was quantified by evaluation of mucosal thickness and leukocyte recruitment as described previously (9). Macrophage counts were confirmed on separate sections by F4/80 staining.

Immunostaining for F4/80

Paraffin-fixed sections were rehydrated, microwaved in Ag retrieval solution (0.01 M citric acid, 0.05 M NaOH [pH 6.0]) for 4 min, and incubated in 3% H₂O₂ in water to block endogenous peroxidases. Sections were blocked in normal goat serum for 30 min (1:10) in PBS and 3% BSA and incubated with anti-mouse F4/80 Ag-pan macrophage marker, BM8 primary Ab (1:300; eBioscience, San Diego, CA), and IgG control (1:300; eBioscience) in PBS and 0.1% BSA for 30 min. Slides were washed with PBS and incubated with FITC mouse anti-rat IgG (1:400; eBioscience) secondary Ab for 30 min, light protected. After PBS washes, sections were coverslipped using Cytoseal containing DAPI (Vector Laboratories, Burlingame, CA) and evaluated by fluorescence microscopy.

Bacterial clearance

The in vivo clearance of *NTHi* from the ME of TNF^{-/-} and WT mice was determined by culturing, on chocolate agar plates, 1 μ l ME effusion from 6–10 MEs per time point and condition on chocolate agar plates. MEs without effusion were scraped using a 1- μ l loop to recover any bacteria on the mucosal surface. CFUs per microliter were assessed after 24 h of culture and analyzed semiquantitatively as described previously (9). The influence of TNF and CCL3 was evaluated by pretreating mice with rCCL3 (50 ng/ml; eBioscience), rCCL3 plus rTNF (50 ng/ml; rTNF: R&D Systems) and an anti-mouse MIP-1 α /CCL3Ab (10 μ g/ml; R&D Systems) 24 h prior to *NTHi* administration. MEs were evaluated for *NTHi* at 3 or 5 d. All analyses were performed in at least duplicate. *NTHi* was verified by Gram-negative staining of CFUs and by negative cultures on hemagglutinin versus chocolate agar plate.

Isolation and culture of peritoneal-derived macrophages

Primary peritoneal macrophages were obtained from six to nine TNF^{-/-} and TLR2^{-/-} mice and control mice (WT) by i.p. injection of 3 ml thioglycolate medium. Cells were harvested 3 d later by peritoneal lavage with cold RPMI 1640, containing 10% FBS, 50 U/ml penicillin, and 50 μ g/ml streptomycin and β -mercaptoethanol, washed with media, enumerated, and seeded into 48-well plates (5 \times 10⁵/well) in triplicates for each condition and time point. For in vitro macrophage/*NTHi* interaction, cells were treated with rTNF (10–100 ng/ml), rCCL3 (10–100 ng/ml), and rTNF plus rCCL3 (each 50 ng/ml) for 24 h in fresh media without antibiotics.

Macrophage phagocytosis and killing assay

Phagocytosis and killing activities of macrophages were assessed using an established in vitro assay (9). Midexponential phase *NTHi* were harvested from fresh cultures and resuspended in PBS. Ten microliters (5 \times 10⁷/well), a titer that does not saturate the cells, was added to give a ratio of bacteria to cells, ~100:1. The tissue culture plates were spun at 1000 rpm for 5 min to enhance contact between the bacteria and macrophages, and infected monolayers were incubated with *NTHi* for 1 h at 37°C. Extracellular bacteria were removed by washing with DMEM. Fresh DMEM containing 10% FCS, macrophage-CSF, and gentamicin (50 μ g/ml) was added to each well to ensure that the remaining extracellular

bacteria were killed, leaving the intracellular bacteria intact, as gentamicin does not penetrate the phagocytes. The minimum concentration of gentamicin required to kill extracellular *NTHi* had previously been determined to be 1 µg/ml. At the end of 1 or 3 h, the cells were rinsed and lysed using 0.5 ml pyrogen-free water followed by aspiration of the lysate five times through a 23-gauge syringe. Lysates were plated on chocolate agar plates and incubated overnight at 37°C in serial dilutions of 1:1 to 1:10⁵ and evaluated by CFUs. Six wells were used per time point and condition. The recovery of bacteria after macrophage treatment with gentamicin for 1 h was used to represent phagocytosis. The ratio of bacteria recovered after gentamicin treatment for 3 h over bacteria recovered posttreatment for 1 h was taken to represent intracellular killing, similar to other published assays (9,32). Bacterial killing percent was calculated as previously described (32) using the following formula: killing % = [1 – (number of CFU in tested wells at 3 h with gentamycin treatment/number of CFU in tested wells 1 h with gentamycin) × 100].

To assure that phagocytosis of *NTHi* by macrophages was not saturated at the bacterial dose used, cultures were also exposed up to 5 × 10⁸ /well, treated with gentamicin for 1 h, and intracellular *NTHi* immediately recovered. Incubation with the 10-fold higher concentration of 5 × 10⁸ *NTHi* resulted in substantially more bacterial uptake than incubation with 5 × 10⁷ *NTHi* (23.5 × 10⁶ versus 0.85 × 10⁵ CFU for WT macrophages; 11 × 10⁶ versus 0.45 × 10⁵ for TNF^{-/-} macrophages). To determine whether the kinetics of intracellular bacterial recovery was affected by TNF deletion and/or CCL3 treatment, intracellular bacterial recovery in macrophages was assessed postincubation with 5 × 10⁸ *NTHi* for 15 or 30 min and 1, 2, 3, 5, and 9 h for both genotypes with or without rCCL3 treatment.

ELISA

Levels of CCL3 and TNF were measured in ME effusions post *NTHi* infection and in the supernatants of macrophage cultures. ME effusions were sampled by extracting fluid from the ME 24 h post *NTHi* inoculation, followed by rinsing with ELISA buffer. The ME effusions, including the rinses, and the supernatants of the cell cultures were analyzed for CCL3 and TNF content by ELISA using the DuoSet ELISA kit (R&D System) and corrected for dilutions. Recombinant proteins were used to calibrate the assay, and the sensitivity of the ELISA was 2 pg/ml for CCL3 and 12 pg/ml for TNF.

Detection of highly reactive oxygen species

Peritoneal macrophage superoxide production was evaluated by detection of highly reactive oxygen species (hROS) using aminophenyl fluorescein, which fluoresces only in the presence of hROS, such as free hydroxyl radical (·OH) and peroxyntrite (ONOO⁻) or major macrophage hROS. Cells were treated with rTNF (50 ng/ml), rCCL3 (50 ng/ml), rTNF plus CCL3 (each 50 ng/ml), or anti-mouse MIP-1α/CCL3 Ab (10 µg/ml). Macrophages were resuspended in modified HBSS containing 10.0 µM HEPES, 1.0 µM MgCl₂, 2.0 µM CaCl₂, and 2.7 µM glucose. Cells were loaded with aminophenyl fluorescein (10 µM; Cell Technology, Columbia, MD) for 1 h at 37°C and infected with 5 × 10⁷ *NTHi*. Luminescence was measured in the live cells, and images were taken by fluorescence microscopy, followed by image analysis with ImageJ (National Institutes of Health, Bethesda, MD) at 15 min. One set of cells was fixed with PFA (4%) in PBS for 20 min and washed with PBS. Postpermeabilization with Triton X-100 (5%; Sigma-Aldrich, St. Louis, MO) in PBS and blocking in FBS (10%) for 20 min, macrophages were stained with DAPI (1:500) for 10 min, rinsed with PBS, and mounted (Vectastain, Vector Laboratories) for additional imaging.

Statistics

One- and two-way ANOVA analysis, followed by Fisher post hoc test with Bonferroni correction for multiple tests, was performed using StatView 5.0 software (JMP-SAS Institute, Cary, NC). Differences between experimental groups were considered significant at $p < 0.05$. The Mann-Whitney U test was used for data that was not normally distributed, as in analyses of ME inflammatory cell numbers, bacterial clearance, and *NTHi* lysate counts of the phagocytosis assay. For purposes of statistical analysis, left and right ears were considered to be independent as described elsewhere (37).

Results

Lack of TNF alters the pathogenesis of OM

To determine the role of TNF in *NTHi*-induced OM, we initially examined the inflammatory response to instillation of *NTHi* into the ME of mice lacking TNF and compared their response to that of WT animals. Prior to inoculation of *NTHi*, the ME mucosa of noninfected $TNF^{-/-}$ mice was significantly thicker than that of their noninfected WT counterparts. WT mice, when exposed to *NTHi*, demonstrated an inflammatory response associated with maximal mucosal thickness 2 d postinfection, with complete recovery noted by day 5. In contrast, in the absence of TNF, the initial increase of mucosal thickness to *NTHi* noted in WT mice was attenuated, but $TNF^{-/-}$ mice displayed marked and persistent mucosal thickening at later times when WT mice have fully recovered from infection (Fig. 1A, 1B). By days 2 and 3, both strains demonstrated a mucosal response of similar magnitude, but distinct differences between WT and $TNF^{-/-}$ animals were noted after day 5, in which $TNF^{-/-}$ mice displayed significantly increased mucosal thickness that persisted through the last time point assessed on day 21.

Persistent OM in $TNF^{-/-}$ mice is associated with significantly decreased early gene expression of IL-1 β , CCL3, and TLR2

To investigate possible mechanisms for the altered inflammatory responses of $TNF^{-/-}$ mice, we assessed expression of genes in the TLR-pathway triggered by *NTHi* in the ME mucosa of WT and $TNF^{-/-}$ mice, at various times during the course of experimental OM (Fig. 1C). *NTHi* triggered the rapid and transient expression of *TNF* mRNA in the WT ME at 6 h, which rapidly declined to low levels after d 2. Expression of the proinflammatory genes *IL-1 β* , *IL-6*, *CCL3*, and *CXCL2* displayed similar kinetics in response to *NTHi* infection in WT mice, showing a rapid increase at 6 h, but more modest increases from day 2 through day 5 (Fig. 1C). Compared to the responses noted in ME mucosa of WT mice, in the ME mucosa of $TNF^{-/-}$ animals, expression of these genes was significantly higher than in WT mice prior to infection (time 0), but strikingly, a rapid fall in their expression was seen post *NTHi* inoculation (Fig. 1C). This was particularly true for the expression of *IL1 β* and *CCL3*. Expression of genes encoding TLR molecules was also altered in response to *NTHi* in $TNF^{-/-}$ as compared with WT mice. In WT mice, *TLR2* gene expression was significantly increased at 6 h and 1 d postinfection, whereas *TLR4* expression was not (Fig. 1C). In contrast, in $TNF^{-/-}$ mice, the genes for both *TLR2* and *TLR4* were expressed at higher levels than in WT mice prior to infection, but mRNA levels for these molecules decreased below the detection threshold soon after *NTHi* challenge.

Absence of TNF is associated with diminished but prolonged neutrophil recruitment, increased and persistent recruitment of macrophages, decreased CCL3 protein expression, and a failure to clear *NTHi* from the ME cavity

The two main effect or cells in clearance of bacteria in this murine model of OM are neutrophils and macrophages. In WT mice, neutrophils were the dominant cell type

identified during initial stages of inflammation, rapidly emigrating into the ME lumen with in 6 h of *NTHi* inoculation. Macrophage accumulation followed at day 2 through day 5 (Fig. 2A, 2B), with smaller numbers of these cells than of neutrophils. By day 10, the ME cavity was cleared of both cell types in WT mice. This classic pattern of inflammatory cell recruitment was significantly altered in the absence of TNF, where the initial recruitment of neutrophils into the ME cavity was strongly compromised, never reaching the peak seen in WT animals. However, neutrophils persisted through day 21 (Fig. 2A). In contrast to WT mice, macrophages were the dominant cell type recruited to the ME of $TNF^{-/-}$ mice (Fig. 2B, 2C), appearing earlier at 6 h and persisting until day 21. Their numbers in the ME were also consistently greater than in WT mice. TNF and CCL3 protein levels were evaluated in ME effusions 1 d post *NTHi* inoculation (Fig. 2D). In WT mice, TNF was below the detection threshold prior to bacterial inoculation and increased to $1.0 (\pm 1.4)$ ng/ml in response to *NTHi*. CCL3 was also undetectable in the naive WT or $TNF^{-/-}$ MEs, but increased to $17.0 (\pm 3.75)$ ng/ml postinoculation with *NTHi* in WT mice but to only $7.5 (\pm 1.25)$ ng/ml in $TNF^{-/-}$ MEs ($p < 0.01$).

Next, we assessed the presence of *NTHi* organisms in the ME cavity of WT and $TNF^{-/-}$ mice (Fig. 2E). Viable *NTHi* were completely cleared by day 5 in WT mice. In $TNF^{-/-}$ mice, however, the bacterial load recovered from the ME cavity was not only consistently and significantly higher than that obtained from WT mice, but also persisted through day 21, the last time point that we examined (Fig. 2E). Thus, the persistent inflammation seen in the ME of $TNF^{-/-}$ mice, which was accompanied with suppressed gene and protein expression of proinflammatory cytokines, chemokines and TLRs, is functionally associated with an altered capacity to clear *NTHi* in murine OM.

Bacterial phagocytosis and intracellular killing by peritoneal macrophages are impaired in the absence of TNF

Because $TNF^{-/-}$ mice failed to clear *NTHi* from ME cavity despite more accumulation of macrophages in the ME lumen, we hypothesized that attenuated macrophage function underlies the aberrant response in the ME of $TNF^{-/-}$ mice. We therefore evaluated the phagocytic and intracellular killing capacity of peritoneal macrophages derived from WT and $TNF^{-/-}$ mice in vitro (Fig. 3A). Compared to macrophages obtained from WT mice, macrophages from $TNF^{-/-}$ animals showed significantly impaired phagocytic function, demonstrated by decreased uptake of *NTHi* at 1 h. Additionally, at 3 h, macrophages from $TNF^{-/-}$ mice exhibited reduced intracellular killing of the bacteria when compared with WT cells (Fig. 3A).

To further explore the mechanism for these functional deficits, we evaluated the expression of genes in macrophages likely involved in their ability to phagocytose and kill *NTHi* (Fig. 3B). Regardless of the genotype, we could not detect gene expression of *CCL3*, *IL-1 β* , or *CXCL2*, known to be produced by activated macrophages and (with the exception of *IL-1 β*) known to be involved in phagocytosis and killing in naive macrophages not exposed to *NTHi*. However, all of these genes were upregulated by *NTHi* in both genotypes, although gene expression of *CCL3* and *IL-1 β* was substantially decreased in $TNF^{-/-}$ macrophages (Fig. 3B). Similar to $TNF^{-/-}$ macrophages, we found decreased expression of *CCL3* and *TNF* mRNA in $TLR2^{-/-}$ macrophages when compared with WT macrophages post *NTHi* infection, suggesting that TLR2 senses *NTHi* in macrophages (Supplemental Fig. 1).

Although mRNA encoding *TLR2* and *TLR4* was detectable prior to *NTHi* challenge in both $TNF^{-/-}$ and WT macrophages, $TNF^{-/-}$ macrophages expressed significantly lower levels of both (Fig. 3B). After *NTHi* challenge, *TLR2* mRNA increased in macrophages of both genotypes, with a significantly higher expression in WT macrophages. As noted in vivo in

the ME mucosa, *TLR4* expression in macrophages strongly decreased after *NTHi* infection in both WT and *TNF*^{-/-} mice.

Exogenous rTNF partially restores phagocytosis and intracellular killing capacity and induces expression of CCL3 in macrophages derived from *TNF*^{-/-} mice

In an attempt to understand the mechanism underlying the impaired function seen in *TNF*^{-/-} animals in vivo and in vitro, we next assessed the effect of the addition of individual mediators upon the ability of macrophages to phagocytose and kill *NTHi*. Macrophages from WT mice responded to rTNF stimulation with a strong and dose-dependent increase in their ability to phagocytose *NTHi* (Fig. 4A). They also showed increased intracellular killing (Fig. 4B). Stimulation of *TNF*^{-/-} macrophages with rTNF significantly increased phagocytic capacity in a dose-dependent manner, but only to a level ~60% of that observed in unstimulated WT mice, with the highest response seen at 50 ng/ml rTNF (Fig. 4A). Similarly, a dose-dependent improvement in bacteriocidal capacity was observed in *TNF*^{-/-} macrophages, but it remained deficient when compared with unstimulated WT even at a dose of 100 ng/ml rTNF (Fig. 4B). To further explore the effects of rTNF on macrophage behavior, we next assessed its effect on the expression of genes known to be increased in macrophages in response to *NTHi*. In the absence of *NTHi*, no *CCL3* mRNA was expressed by macrophages of either genotype in response to any concentration of exogenous rTNF. However, rTNF resulted in an approximate doubling of *NTHi*-triggered increase of *CCL3* expression in macrophages from both WT and *TNF*^{-/-} mice, with consistently lower expression in *TNF*^{-/-} macrophages (Fig. 4C). In response to rTNF, a modest and comparable augmentation of *NTHi*-induced mRNA encoding *CXCL2* was noted in both cell types. *IL-1 β* mRNA was modestly augmented by rTNF in WT but not in *TNF*^{-/-} macrophages. rTNF also suppressed the *NTHi*-induced *TLR2* mRNA expression in both genotypes, but did not alter the expression of mRNA for *TLR4* seen in *TNF*^{-/-} mice (not shown).

Interestingly, the level of CCL3 protein as measured by ELISA in both WT and *TNF*^{-/-} macrophage supernatants was significantly increased at 6 and 24 h in response to *NTHi*, although the increase was less in *TNF*^{-/-} macrophages (Fig. 4D). However, CCL3 protein was increased by exposure to 10–100 ng/ml rTNF in macrophages from *TNF*^{-/-} mice at 24 h to levels similar to those observed in WT macrophages in response to *NTHi* alone (Fig. 4D).

Exogenous rCCL3 improves phagocytosis and killing by macrophages from *TNF*^{-/-} mice

The finding that *CCL3* gene expression was augmented in macrophages from WT and *TNF*^{-/-} animals and that CCL3 protein levels were fully restored in macrophages from *TNF*^{-/-} mice by exogenous rTNF led us to examine the role of CCL3 in modulating the phagocytic and bacteriocidal function of macrophages. Exogenous rCCL3 strongly enhanced phagocytosis of *NTHi* by macrophages from WT mice, but only modestly enhanced intracellular killing. In *TNF*^{-/-} macrophages, the addition of graded amounts of CCL3 restored both phagocytosis and killing functions in a dose-dependent manner to a level indistinguishable from those of WT mice responding to *NTHi* alone (Fig. 5A, 5B). Although the response of *TNF*^{-/-} macrophages was less than that of WT cells, rCCL3 was more effective in restoring these functions than the addition of an equivalent concentration of rTNF (Fig. 5B). The addition of both rCCL3 and rTNF (50 ng/ml each) to *TNF*^{-/-} macrophages proved most potent in augmenting phagocytosis and bacterial killing by these cells (Fig. 5A, 5B).

When WT and *TNF*^{-/-} macrophages were incubated with *NTHi* for various periods, bacterial recovery from macrophages was low at 15 and 30 min, but was substantially

increased ($p < 0.001$) at 1 h (Supplemental Fig. 2). When bacteria were left on the cells for 2 or 3 h, recovery of bacteria declined sharply in WT cells. However, the peak of bacterial recovery was observed at 2 h in $TNF^{-/-}$ macrophages, suggesting delayed phagocytosis and impaired intracellular killing in the absence of TNF. *NTHi* recovery declined slightly at 3 h in $TNF^{-/-}$ cells. Significantly fewer bacteria were recovered at 2 to 3 h from $TNF^{-/-}$ macrophages stimulated with CCL3 ($p < 0.001$), consistent with the enhanced intracellular killing shown in Fig. 5B. At 5 and 9 h, bacteria were recovered only from $TNF^{-/-}$ macrophages without CCL3 stimulation (not shown).

In contrast to rTNF (Fig. 4C), exogenous rCCL3 significantly induced the expression of *CXCL2* mRNA in both genotypes, whereas *IL-1 β* gene expression was augmented by rCCL3 in WT but not in $TNF^{-/-}$ cells, similar to the effect of rTNF. rCCL3 did not have any effects on *TLR2* or *TLR4* mRNA expression in both genotypes, in contrast to *TLR2* mRNA suppression by rTNF.

rCCL3 and rTNF induce hROS production in *NTHi*-exposed macrophages

rCCL3 stimulated the intracellular killing in $TNF^{-/-}$ macrophages significantly more than rTNF and did so to a level seen in WT macrophages exposed to *NTHi* in the absence of exogenous cytokines. To further elucidate the nature of the bacteriocidal defects in $TNF^{-/-}$ mice, we investigated the production of hROS by macrophages of WT and $TNF^{-/-}$ mice in response to *NTHi* in the presence or absence of rTNF or rCCL3, alone or in combination. Indeed, *NTHi*-infected cells, but not uninfected ones, expressed cytoplasmic hROS at 15 min postexposure to bacteria, although significantly fewer hROS-positive $TNF^{-/-}$ macrophages were observed than in WT macrophages (Fig. 6). Addition of rTNF or rCCL3 alone as well as their combined addition further increased the production of hROS in *NTHi* exposed macrophages of both genotypes. The hROS production of $TNF^{-/-}$ macrophages was restored by rTNF to the level seen in WT cells in response to *NTHi* alone. Generation of hROS was augmented even more in $TNF^{-/-}$ macrophages in response to exogenous rCCL3 given alone or with rTNF.

rCCL3 improves the clearance of *NTHi* from the ME of $TNF^{-/-}$ animals in vivo

To extend our in vitro observations to the in vivo model of OM, we treated WT and $TNF^{-/-}$ mice with rCCL3, rCCL3 plus rTNF, or with a neutralizing Ab against CCL3 24 h preinfection with *NTHi*. The levels of rCCL3 and rTNF employed for this one-time administration (50 ng/ml) were substantially higher than those we observed in either the WT or $TNF^{-/-}$ ME 24 h post *NTHi* inoculation (Fig. 2D) and were chosen to ensure that sufficient mediator was present throughout the experiment. Anti-CCL3 pretreatment impaired the ability of WT mice to clear *NTHi* from the ME at 3 and 5 d when compared with buffer-treated control animals (Fig. 7). $TNF^{-/-}$ mice, which normally fail to clear bacteria from the ME (Fig. 2A), showed significantly improved bacterial clearance at days 3 and 5 after pretreatment of their MEs with rCCL3 alone. This protective effect was further enhanced by pretreatment with the combination of rCCL3 and rTNF (Fig. 7), such that the antibacterial defects noted in $TNF^{-/-}$ animals were fully recovered.

Discussion

Antimicrobial activity mediated by innate immunity is central to the host response to infection. Deficiencies in key innate immune signaling molecules, such as TLR2 and MyD88, have been shown experimentally to increase the severity of infections and to be associated with impaired phagocytosis and killing of bacteria (9,10,39,40). Moreover, mutations and polymorphisms in the TLR/MyD88 signaling axis have been associated with increased incidence and severity of infections in humans (12,29). We have recently

demonstrated that TNF, a downstream mediator of innate immunity, is a major target of signaling through TLR2 and TLR4 in *NTHi*-induced OM and is strongly downregulated in the absence of either TLR (10). TNF is known to play both positive and negative feedback roles in TLR signaling through its ability to regulate the expression of TLRs and other inflammatory genes via NF- κ B (41,42).

Because TNF expression is increased during normal responses to experimental OM in mice, and its expression is suppressed in animals not able to properly clear OM due to lack of TLR signaling molecules, we have assessed the role of TNF during OM in mice. Infection of the MEs of TNF^{-/-} mice resulted in aberrant and prolonged inflammation as manifested by thickening of the ME mucosa (Fig. 1A, 1B) and impaired clearing of bacteria from the ME (Fig. 2E). Interestingly, this was accompanied by a dramatic decrease of proinflammatory genes that are normally increased during *NTHi* infection in WT mice (Fig. 1C). Recruitment of polymorphonuclear leukocytes (Fig. 2A) to the ME in response to *NTHi* was significantly reduced in TNF^{-/-} animals, whereas, in contrast, that of macrophages occurred earlier and persisted for much longer than in WT animals (Fig. 2B). Despite the prolonged presence of inflammatory leukocytes, bacteria were not cleared from the ME in TNF^{-/-} mice. Moreover, macrophages derived from TNF^{-/-} animals were defective in their ability to phagocytose and kill *NTHi* (Fig. 3).

As bacteria are known to drive inflammation in this model, the inefficient killing of *NTHi* noted in TNF^{-/-} mice may have, at least in part, mediated the prolonged mucosal thickening and cellular infiltration noted in these animals. The early influx of mononuclear leukocytes seen in TNF^{-/-} animals may have been mediated by CXCL2, because gene expression of this chemokine was elevated in TNF^{-/-} animals prior to *NTHi* infection (Fig. 1C). The failure of macrophages to clear from the ME, despite reduced CCL3 and a probable reduction in macrophage migration inhibiting factor (known to be induced by TNF) (43), is presumably due to persistence of *NTHi*. The continued presence of macrophages in the MEs of TNF^{-/-} mice during experimental OM is reminiscent of that seen in a severe colitis model in this strain (44) but differs from the reduced macrophage infiltration of the lung seen in mycobacterial infection of mice lacking TNF (45).

The absence of TNF was also accompanied by diminished expression of genes encoding *TLR2*, *TLR4*, *CCL3*, and *IL-1 β* in response to *NTHi* in the ME mucosa (Fig. 1C). TLR2 is an important receptor for bacterial pathogen-associated molecular patterns found in *NTHi* (18), whereas CCL3 is a critical mediator in the recruitment and activation of PMNs and macrophages (31,46-48). In any event, the alterations seen in TNF^{-/-} mice in response to ME bacterial infection are consistent with an important role for TNF in regulating and amplifying responses to TLR signaling. As TNF, the β form of pro-IL-1 signaling, and CCL3 activation are important in neutrophil recruitment in other models (46,47), their dysregulation in TNF^{-/-} animals provides an additional mechanism for the altered inflammatory responses reported in this study.

Critical to the persistent inflammation and bacterial presence in this model is the impaired function of macrophages, decreased rate of phagocytosis, and compromised ability to kill intracellular bacteria. Defects in macrophage function identified in TNF^{-/-} mice appear to be related to the effect of TNF on downstream effector molecules. In the absence of TNF, the ability of *NTHi* to activate macrophages in vitro was reduced, as exemplified by their diminished expression of genes for the downstream molecules CCL3 and the β form of pro-IL-1 and for the upstream receptor TLR2 (Fig. 3). Such reduced expression would be expected to impair the in vivo antimicrobial function of macrophages from TNF-deficient animals and to reduce recruitment of PMNs into the ME cavity. Reduced production of hROS in macrophages lacking TNF would be expected to decrease bacterial killing.

The addition of exogenous rTNF to macrophages derived from TNF^{-/-} animals partially restored their ability to phagocytose and kill *NTHi* (Fig. 4A, 4B). The inability of TNF to fully restore these functions suggests long-term changes in the cellular response to this cytokine perhaps mediated by stable changes in receptors or downstream targets. This idea is further supported by the altered expression of genes for *TLR2* and *CXCL2* in response to rTNF in TNF^{-/-} macrophages compared with WT cells (Fig. 4C).

rTNF was able to enhance CCL3 gene and protein expression in TNF^{-/-} macrophages, but not to the extent seen in cells derived from WT mice (Fig. 4C, 4D). This may be a critical difference, as CCL3 treatment restored most of the responses seen in WT cells: phagocytosis, bacterial killing, gene expression, and hROS production were all markedly responsive to exogenous rCCL3 in TNF^{-/-} macrophages (Figs. 5, 6). As with rTNF, the fact that TNF^{-/-} cells were less responsive to rCCL3 than WT cells suggests stable changes in the receptor and/or signaling repertoires of these cells. It should also be noted that the effect of rCCL3 on *NTHi* phagocytosis was not simply an effect on kinetics, because the relative increase in *NTHi* uptake over time was similar in untreated and in rCCL3-treated WT macrophages.

These latter findings support the hypothesis that TNF acts through this downstream chemokine in regulating macrophage function. The results also demonstrate that macrophages from TNF^{-/-} mice are capable of normal function, only requiring appropriate activation to fulfill their role in control of bacterial infection. The functional importance of CCL3 to macrophages is illustrated by the restoration of hROS production and *NTHi* killing capacity of TNF^{-/-} macrophages by rCCL3 alone or in combination with rTNF (Figs. 5B, 6A). Of broader significance is the ability of rCCL3, alone or in combination with rTNF, to rescue the ability of TNF^{-/-} mice to clear *NTHi* in vivo and the ability of the Ab to CCL3 to impair clearance of bacteria from WT mice. These findings identify CCL3 as a critical mediator of TNF function and an essential component of the normal response to bacterial infection in the ME and perhaps in other sites as well.

Enhanced bacterial phagocytosis and killing by human and murine macrophages in response to rCCL3 has been previously reported (32,49). However, our studies provide evidence that induction of CCL3 plays a central role in macrophage biology. It also appears likely that full expression of TNF function requires a critical level of CCL3.

As defects in TLR signaling, including IL-1R-associated kinase 4 and MyD88 deficiency (50,51) or disturbed TNF production (13,29,30), increase susceptibility to infections in children, the importance of innate immune signaling in host defense against bacterial disease is underscored (52). We have reported previously that the absence of TLR2, TLR4, or MyD88 (9,10) results in defective bacterial clearance and persistent OM in mice, similar to that seen in TNF^{-/-} animals. Because TNF is a major downstream product of TLR/MyD88 signaling, it is therefore possible that CCL3 would rescue the phenotypes of these mice as well. This idea is supported by the impaired expression of *TNF* and *CCL3* mRNA by TLR2^{-/-} macrophages, observed in response to *NTHi* (Supplemental Fig. 1). These data suggest that CCL3 is a crucial component of *NTHi*-signaling and that many innate immune responses to *NTHi* may be mediated by a TLR2/TNF-mediated CCL3 response.

In summary, we show that in a murine model of OM, the absence of TNF is associated with aberrant inflammatory responses, defective macrophage function, and prolonged OM. rCCL3 can rescue the abnormal response of TNF^{-/-} mice both in vitro and in vivo and also enhance the function of WT macrophages, suggesting that this chemokine is a central effector in OM and a potential therapeutic target for persistent disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Shizuo Akira and Eyal Raz for TLR2^{-/-} mice, Eduardo Chavez for mouse colony maintenance, and Julie Lightner for editorial help.

This work was supported by Grants DC000129 (to A.F.R.), DC006279 (to S.I.W.), AR052728, and AI052453 (to R.G.) from the National Institutes of Health, Veteran Affairs Merit Awards (to A.F.R. and R.G.), and Grant EU 120/1-1 from the German Research Foundation (to S.E.).

Abbreviations used in this paper

hROS	highly reactive oxygen species
ME	middle ear
NTHi	nontypeable <i>Haemophilus influenzae</i>
OM	otitis media
PFA	paraformaldehyde
qRT-PCR	quantitative RT-PCR
WT	wild-type

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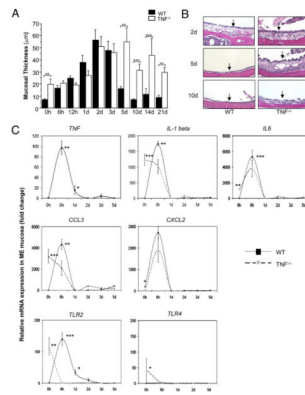
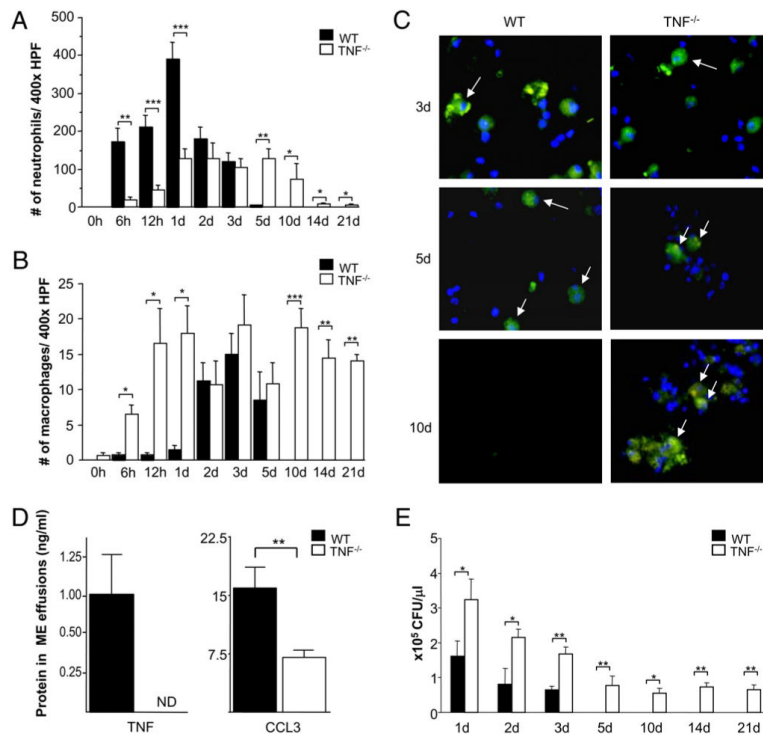


FIGURE 1.

TNF^{-/-} mice develop persistent mucosal thickness and decreased initial inflammatory gene expression during *NTHi*-induced OM. *A*, Time course of mucosal thickness in response to *NTHi* in WT (closed bars) and TNF^{-/-} mice (open bars). Results are expressed as means \pm SEM of six MEs. *B*, Histologic changes in MEs of WT (*left panels*) and TNF^{-/-} (*right panels*) mice at 2 d (*top panels*), 5 d (*middle panels*), and 10 d (*lower panels*) postinstillation of *NTHi* in the ME (original magnification $\times 100$). Arrows denote mucosa. *C*, Relative mRNA expression measured by qRT-PCR of selected downstream cytokine/chemokine genes and *TLR2* and *TLR4* in ME mucosa prior to and post *NTHi* instillation into the ME of WT (solid lines) and TNF^{-/-} (broken lines) mice. Target transcripts of WT and TNF^{-/-} MEs were normalized to GAPDH and compared with uninfected mucosa of WT animals (0 h). Experiments were performed in triplicate and evaluated using two-way ANOVA and Fisher post hoc test with Bonferroni correction (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, for this and subsequent figures).

**FIGURE 2.**

TNF^{-/-} MEs display impaired bacterial clearance and neutrophil recruitment, but increased macrophage presence post *NTHi* infection. The numbers of neutrophils (A) and macrophages (B) in the ME cavity of WT (closed bars) and TNF^{-/-} (open bars) mice per ×400 high-power field of H&E and immunostained specimens ($n = 6$). C, Immunohistochemical identification of macrophages in ME of WT (left panel) and TNF^{-/-} (right panel) mice at 3, 5, and 10 d postinstillation of *NTHi* (original magnification ×400). Macrophages were stained with monoclonal anti-mouse F4/80 Ab and FITC mouse anti-rat IgG (green) and nuclei are stained with DAPI (blue). Autofluorescence (yellow) represent erythrocytes in the ME cavity. Arrows denote macrophages. D, ME cell effusion was assessed for TNF and CCL3 protein by ELISA 1 d post *NTHi*-inoculation from WT and TNF^{-/-} mice. Experiments were performed in triplicate and expressed as means ± SEM from individual mice. E, Bacterial presence as CFUs detected in the MEs of WT and TNF^{-/-} mice postinstillation of *NTHi*. CFUs per microliter sample loop identified after 24 h of bacterial culture are displayed ($n = 6$).

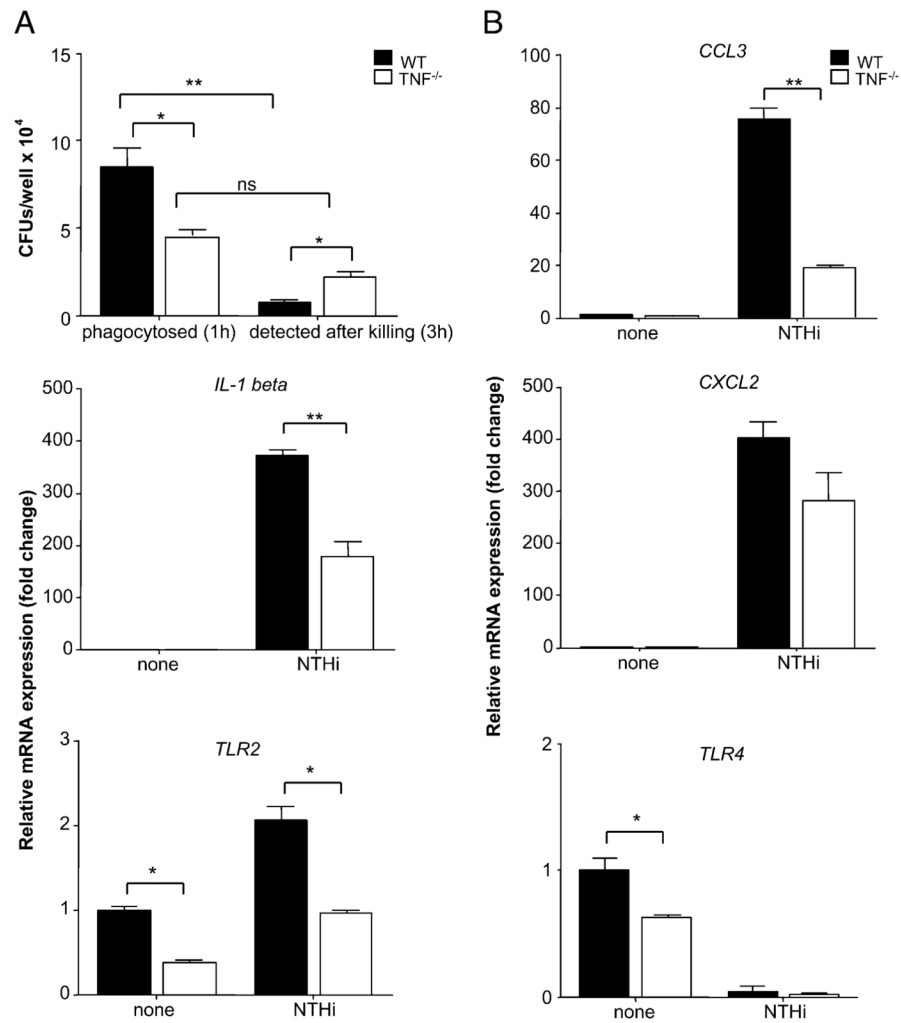


FIGURE 3. Phagocytosis and killing of bacteria by TNF^{-/-} macrophages is impaired and is associated with decreased expression of the genes for *CCL3*, *IL-1β*, and *TLR2*. **A**, Phagocytosis and killing of *NTHi* bacteria was assessed in peritoneal macrophages from WT (closed bars) and TNF^{-/-} (open bars) mice. Bacteria remaining after 1 h (phagocytosis) and 3 h (killing) were quantified by colony counting. **B**, Expression of genes for *CCL3*, *CXCL2*, *IL-1β*, *TLR2*, and *TLR4* after 6 h exposure of macrophages from WT and TNF^{-/-} mice to *NTHi* was determined by qRT-PCR. Experiments were performed in triplicate and expressed as means ± SEM. Data are representative of three independent experiments.

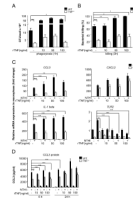
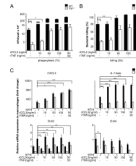


FIGURE 4.

rTNF augments phagocytic and killing activity of TNF^{-/-} macrophages and induces an *NTHi*-dependent increase of CCL3 expression. Peritoneal-derived macrophages from WT (closed bars) and TNF^{-/-} (open bars) mice were stimulated in vitro with varying concentrations of rTNF for 24 h before infection with *NTHi*. Phagocytosis (A) and killing (B) of *NTHi* was assessed. Killing is displayed as the percentage of phagocytosed *NTHi*. C, Expression of genes for selected cytokines, chemokines, and *TLR2* by macrophages from WT and TNF^{-/-} mice after 6 h exposure to *NTHi* in the presence or absence of varying concentrations of rTNF. Gene expression by qRT-PCR was normalized to GAPDH and compared using WT at 0 h as baseline. D, CCL3 protein assessed by ELISA (pg/ml) in supernatant of macrophages from WT and TNF^{-/-} mice at 6 and 24 h postexposure to *NTHi* in the presence or absence of varying concentrations of rTNF. Experiments were performed in triplicate and expressed as means \pm SEM from individual mice.

**FIGURE 5.**

rCCL3, alone or with rTNF, enhances phagocytosis and killing of *NTHi* by macrophages of $TNF^{-/-}$ mice and induces gene expression of *CXCL2*. Phagocytosis (A) and killing (B) of *NTHi* by macrophages from WT (closed bars) and $TNF^{-/-}$ (open bars) mice in the presence or absence of varying concentrations of rCCL3 alone or in combination with 50 $\mu\text{g/ml}$ rTNF. C, Expression of selected genes from macrophages of WT and $TNF^{-/-}$ mice from cell lysates obtained 6 h postexposure to *NTHi* in the presence or absence of rCCL3 with or without 50 μg rTNF. mRNA levels were assessed using qRT-PCR and normalized to GAPDH. Data are expressed as means \pm SEM and represent one of three independent experiments, each performed in triplicate.

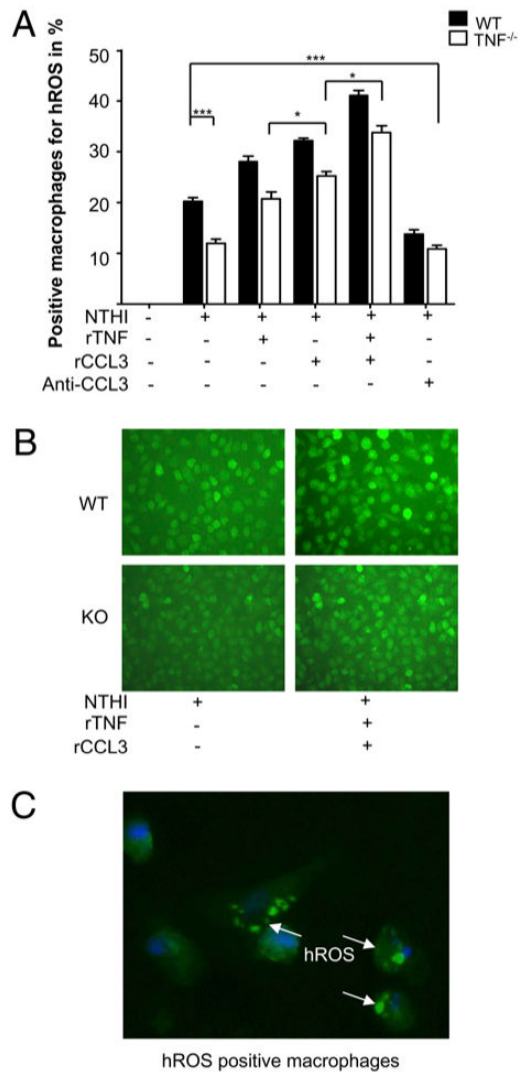
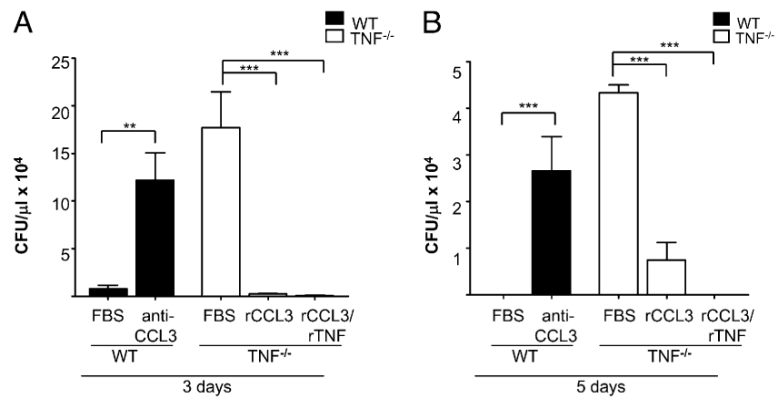


FIGURE 6. rCCL3 and rTNF restore impaired hROS production by TNF^{-/-} macrophages in response to *NTHi*. **A**, The percentage of macrophages from WT (closed bars) and TNF^{-/-} (open bars) mice expressing hROS before and after 15 min incubation with *NTHi* in the presence or absence of 50 μ g rCCL3 or rTNF or both or in the presence of Ab to CCL3. **B**, Photomicrographs of hROS production by macrophages from WT (*top panels*) and TNF^{-/-} (*lower panels*) mice in response to *NTHi* with (*right panels*) and without (*left panels*) rTNF plus rCCL3. Original magnification $\times 100$. **C**, Fluorescent localization of hROS (green) in the cytoplasm of WT macrophages exposed to *NTHi* for 15 min. Nuclei were stained with DAPI (blue). Original magnification $\times 400$.

**FIGURE 7.**

Microbial clearance from the ME of WT and TNF^{-/-} mice in vivo in the presence or absence of rCCL3 alone or in combination with rTNF. The presence of bacteria was quantified in MEs of WT (closed bars) and TNF^{-/-} (open bars) mice at 3 d (A) and 5 d (B) postexposure to *NTHi* in the presence of diluent control (FBS) or anti-rCCL3 (WT mice) or rCCL3 alone or together with rTNF (TNF^{-/-} mice). Data are expressed as means ± SEM.